

EXPRESS MAIL NUMBER: EL 894 013 957 US

PATENT APPLICATION

of

Carl T. Wittwer

For

**AUTOMATED ANALYSIS OF REAL-TIME NUCLEIC ACID AMPLIFICATION**

Attorney Docket 7475-70049

7475-70049

**AUTOMATED ANALYSIS OF REAL-TIME NUCLEIC ACID AMPLIFICATION**Cross-Reference to Related Application

This application is a continuation-in-part of U.S. Patent Application No. 09/391,811, filed September 8, 1999, which claims priority under 35 U.S.C. § 119 (e) to U.S. Provisional Application No. 60/131,256, filed April 27, 1999, which is expressly incorporated by reference herein.

Field of the Invention

The present invention relates to a method of analyzing a sample for the presence of a nucleic acid. More particularly, the present invention is directed to an automated method for detecting and reporting the presence of a predetermined nucleic acid in a sample using polymerase chain reaction and a fluorescent detecting entity.

Background and Summary of the Invention

Amplification of DNA by polymerase chain reaction (PCR) is a technique fundamental to molecular biology. Nucleic acid analysis by PCR requires sample preparation, amplification, and product analysis. Although these steps are usually performed sequentially, amplification and analysis can occur simultaneously. DNA dyes or fluorescent probes can be added to the PCR mixture before amplification and used to analyze PCR products during amplification. Sample analysis occurs concurrently with amplification in the same tube within the same instrument. This combined approach decreases sample handling, saves time, and greatly reduces the risk of product contamination for subsequent reactions, as there is no need to remove the samples from their closed containers for further analysis. The concept of combining amplification with product analysis has become known as "real time" PCR. See, for example, U.S. Patent No. 6,174,670, incorporated herein by reference.

Monitoring fluorescence each cycle of PCR initially involved the use of ethidium bromide. Higuchi R, G Dollinger, PS Walsh and R. Griffith, Simultaneous amplification and detection of specific DNA sequences, Bio/Technology 10:413-417, 1992; Higuchi R, C Fockler G Dollinger and R Watson, Kinetic PCR analysis: real time monitoring of DNA amplification reactions, Bio/Technology 11:1026-1030, 1993. In

that system fluorescence is measured once per cycle as a relative measure of product concentration. Ethidium bromide detects double stranded DNA; if template is present fluorescence intensity increases with temperature cycling. Furthermore, the cycle number where an increase in fluorescence is first detected increases inversely proportionally to the log of the initial template concentration. Other fluorescent systems have been developed that are capable of providing additional data concerning the nucleic acid concentration and sequence.

While PCR is an invaluable molecular biology tool, the practical implementation of real time PCR techniques has lagged behind the conceptual promise. Currently available instrumentation generally does not actually analyze data during PCR; it simply acquires the data for later analysis. After PCR has been completed, multiple manual steps are necessary to analyze the acquired data, and human judgment is typically required to provide the analysis result. What is needed is a system for automating data acquisition and analysis so that no user intervention is required for reporting the analytical results. Thus, when the temperature cycling in a polymerase chain reaction amplification is complete, the system software is automatically triggered and the results, for example, the presence or absence of a given pathogen, are immediately displayed on screen. Algorithms for detection, quantification, and genotyping are needed. Moreover, initiation of the analysis algorithm can be implemented prior to completion of temperature cycling. Data processing can occur during amplification and concomitant analysis results can be used to modify temperature cycling and to acquire additional data during the latter stages of the amplification procedure to optimize amplification protocol and data quality.

A major problem in automating PCR data analysis is identification of baseline fluorescence. Background fluorescence varies from reaction to reaction. Moreover, baseline drift, wherein fluorescence increases or decreases without relation to amplification of nucleic acids in the sample, is a common occurrence. Prior attempts to automate amplification data analysis involved setting the baseline fluorescence as that measured at one or more predetermined early cycle numbers. This technique accounts for the variation in background fluorescence, but it does not compensate for baseline drift. Without compensation for baseline drift, automated amplification data analysis can easily provide both false negative and false positive results.

Thus, in one embodiment a method for determining the presence of a nucleic acid in a sample is provided, said method comprising the steps of providing a fluorescent entity capable of indicating the presence of the nucleic acid and capable of providing a signal related to the quantity of the nucleic acid, amplifying the nucleic acid through a plurality of amplification cycles in the presence of the fluorescent entity, measuring fluorescence intensity of the fluorescent entity at each of the plurality of amplification cycles to produce a fluorescent value for each cycle related to the quantity of the nucleic acid present at each cycle, generating a plot wherein the fluorescent values are recorded for each amplification cycle, performing a confidence band analysis on the plot to generate a positive or negative call, and if the call is positive, confirming the positive call by a melting temperature analysis. The melting profile for the melting temperature analysis may be obtained by monitoring fluorescence between extension and denaturation during one of the amplification cycles, by monitoring fluorescence between annealing and denaturation during one of the amplification cycles, or in a separate melting process subsequent to amplification.

In another embodiment, an automated method for determining the presence of a nucleic acid is provided, comprising the steps of placing a sample into a container containing a fluorescent entity capable of indicating the presence of the nucleic acid and capable of providing a signal related to the quantity of the nucleic acid, placing the container into a device for amplifying the nucleic acid through a plurality of amplification cycles in the presence of the fluorescent entity, measuring fluorescence intensity of the fluorescent entity at each of the plurality of amplification cycles to produce a fluorescent value for each cycle related to the quantity of the nucleic acid present at each cycle, generating a plot wherein the fluorescent values are recorded for each amplification cycle, calculating slopes of segments of the plot using a plurality of the fluorescent values, using the segment slopes of the plot to establish a baseline fluorescence region by generating a slope value for each of a plurality of the amplification cycles, and establishing the baseline fluorescence region comprising an interval of cycles that includes the amplification cycle with the slope value having an absolute value closest to zero, outputting a positive result if the fluorescence value of a selected amplification cycle is outside the baseline fluorescence region, and confirming the positive result by melting temperature analysis.

In yet another aspect of this invention a device is provided for determining the presence of a predetermined nucleic acid in a sample comprising an instrument for temperature cycling to amplify the nucleic acid, a fluorimeter for detecting fluorescence during amplification of the nucleic acid, the fluorescence obtained from a fluorescent  
5 entity capable of providing a signal related to the quantity of the nucleic acid, wherein the fluorimeter measures fluorescence intensity of the fluorescent entity at each of a plurality of amplification cycles, and wherein the fluorimeter measures fluorescence intensity of the fluorescent entity to obtain a melting profile of the nucleic acid, and a processor for performing analysis routines, wherein the processor is programmed to generate a plot of  
10 the fluorescent values verses amplification cycle, to perform a confidence band analysis on the plot to generate a positive or negative call, and to perform melting temperature analysis to confirm a positive call.

Additional features of the present invention will become apparent to those skilled in the art upon consideration of the following detailed description of preferred  
15 embodiments exemplifying the best mode of carrying out the invention as presently perceived.

#### Brief Description of the Drawings

Figs. 1a-l show a comparison of three fluorescence monitoring schemes,  
20 (Figs. 1a, d, g, j) dsDNA dye, (Figs. 1b, e, h, k) exonuclease probe, and (Figs. 1c, f, i, l) hybridization probe, for PCR amplification, wherein each scheme is illustrated (Figs. 1a-c) before amplification and (Figs. 1d-f) after amplification, and fluorescence values are shown (Figs. 1g-i) once during each cycle of PCR and (Figs. 1j-l) continuously during PCR.

25 Fig. 2 is a graph illustrating logistic growth.

Figs. 3a-f show a comparison of various cycle-verses-fluorescence curve types.

Fig. 4 illustrates a sliding window analysis for determining the slope of the fluorescence-verses-cycle number graph at each cycle.

30 Fig. 5 shows typical fluorescence verses amplification cycle graphs for (A) a negative sample and (B) a positive sample.

Fig. 6 also shows typical amplification graphs wherein (A) shows fluorescence verses amplification cycle, (B) is the first derivative of fluorescence verses amplification cycle, and (C) is the second derivative of fluorescence verses amplification cycle.

Figs. 7-11 show the results for various samples wherein open white circles represent the fluorescence measurement at each cycle, open black circles represent the first derivatives, closed black circles represent second derivatives, large black circles connected by lines represent the points contributing to the baseline calculation, and the horizontal lines illustrate the baseline region. Figs. 7 and 8 illustrate positive results, while Figs. 9-11 illustrate negative results.

#### Detailed Description of the Invention

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

As used herein, "nucleic acid," "DNA," and similar terms also include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention.

As used herein, "fluorescence resonance energy transfer pair" or "FRET pair" refers to a pair of fluorophores comprising a donor fluorophore and acceptor fluorophore, wherein the donor fluorophore is capable of transferring resonance energy to the acceptor fluorophore. In other words the emission spectrum of the donor fluorophore overlaps the absorption spectrum of the acceptor fluorophore. In preferred fluorescence resonance energy transfer pairs, the absorption spectrum of the donor fluorophore does not substantially overlap the absorption spectrum of the acceptor fluorophore.

As used herein, "FRET oligonucleotide pair" refers to a pair of oligonucleotides, each labeled with a member of a fluorescent resonance energy transfer pair, wherein hybridization to complementary target nucleic acid sequences brings the fluorescent entities into a fluorescence resonance energy transfer relationship.

The present invention is directed to a method of analyzing a sample for the presence of a nucleic acid wherein the sample is amplified, preferably using PCR, in

the presence of a fluorescent probe capable of detecting the presence of the nucleic acid sample. A baseline region is determined by comparing the fluorescence at various amplification cycles, and the fluorescence at each of various amplification cycles is compared to the baseline region to determine whether the fluorescence measurements fall outside of that baseline region. In an alternative embodiment, various tests are performed on the fluorescent data acquired during amplification, each of which test produces a numeric score. The scores are then used to determine a composite value, and a call is made based on that value.

Many different probes have recently become available for monitoring PCR. Although not sequence specific, double stranded DNA (dsDNA) specific dyes can be used in any amplification without the need for probe synthesis. Such dyes include ethidium bromide and SYBR™ Green I. With dsDNA dyes, product specificity can be increased by analysis of melting curves or by acquiring fluorescence at a high temperature where nonspecific products have melted. Ririe KM, Rasmussen RP and CT Wittwer, Product differentiation by analysis of DNA melting curves during the polymerase chain reaction, *Anal. Biochem.* 245:154-160, 1997; Morrison TB, J&J Weis and CT Wittwer, Quantification of low copy transcripts by continuous SYBR Green I monitoring during amplification, *BioTechniques* 24:954-962, 1998.

Oligonucleotide probes can also be covalently labeled with fluorescent molecules. Hairpin primers (Sunrise™ primers), hairpin probes (Molecular Beacons™) and exonuclease probes (TaqMan™) are dual-labeled oligonucleotides that can be monitored during PCR. These probes depend on fluorescence quenching of a fluorophore by a quencher on the same oligonucleotide. Fluorescence increases when hybridization or exonuclease hydrolysis occurs.

An illustrated probe design employs two oligonucleotides, each labeled with a fluorescent probe. Hybridization of these oligonucleotides to a target nucleic acid brings the two fluorescent probes close together to allow resonance energy transfer to occur. Wittwer CT, MG Herrmann, AA Moss and RP Rasmussen, Continuous fluorescence monitoring of rapid cycle DNA amplification, *BioTechniques* 22:130-138, 1997. These hybridization probes require only a single fluorescent label per probe and are easier to design and synthesize than dual labeled probes. Acceptable fluorophore pairs for use as fluorescent resonance energy transfer pairs are well known to those

skilled in the art and include, but are not limited to, fluorescein/rhodamine, phycoerythrin/Cy7, fluorescein/Cy5, fluorescein/Cy5.5, fluorescein/LC Red 640, and fluorescein/LC Red 705. Donor-quencher FRET oligonucleotide pairs may also be employed, wherein fluorescence of the donor fluorophore is quenched by the quencher fluorophore when the two fluorescent probes are brought close together. It is understood that when donor-quencher FRET oligonucleotide pairs are used, the fluorescence values, and hence all maximum and minimum values, will be the inverse as described below.

Another type of hybridization probe, a “single-labeled oligonucleotide probe,” employs an oligonucleotide probe wherein each probe is constructed of a single oligonucleotide and a single fluorescent dye. The oligonucleotide probes are constructed such that hybridization of the probe to a target sequence affects the fluorescent emission of the fluorescent dye. Single-labeled oligonucleotide probes may employ various probe designs. In one design, hybridization of the probe to the target sequence places the fluorescent dye in close proximity to a guanine residue, with resultant quenching of fluorescent emission. In another embodiment, the fluorescent entity replaces a base in the oligonucleotide probe structure, and upon hybridization this “virtual nucleotide” is placed in a complementary position to a G residue, with resultant quenching of fluorescence. In other embodiments, probes are constructed such that hybridization results in an increase in fluorescent emission. In one such embodiment, the fluorescent entity is attached to a G residue, with increased fluorescence upon hybridization. Further information on single-labeled oligonucleotide probe design is found in U.S. Patent Application No. 09/927,842, filed August 10, 2001, herein incorporated by reference. As with the donor-quencher FRET oligonucleotide pairs, when fluorescent quenching indicates hybridization, the fluorescence values, and hence all maximum and minimum values, will be the inverse as described below

SYBR™ Green I, exonuclease probe, and hybridization probe designs are shown in Figs. 1a-l. For each design, schematics both before (Figs. 1a-c) and after (Figs. 1d-f) amplification are shown, as well as cycle verses fluorescence amplification plots of positive and negative controls (Figs. 1g-i), and temperature verses fluorescence plots from continuous monitoring (Figs. 1j-l). SYBR Green I fluorescence increases as more dsDNA is made (Figs. 1a, d, g, j). Because the dye is not sequence specific, a negative control also increases in fluorescence during later cycles as primer dimers are formed. In



Figs. 1b, e, h, k, dual-labeled fluorescein/rhodamine probes are cleaved during polymerase extension by 5'-exonuclease activity, separating the fluorophores and increasing the fluorescein emission. The signal generated is cumulative and the fluorescence continues to increase even after the amount of product has reached a plateau. Figs. 1c, f, i, l show use of a FRET oligonucleotide pair wherein two probes hybridize next to each other, one labeled 3' with fluorescein and the other labeled 5' with Cy5. As product accumulates during PCR, fluorescence energy transfer to Cy5 increases. The fluorescence of hybridization probes decreases at high cycle number because of probe/product competition.

Standard instruments for PCR complete 30 cycles in about two to four hours. A preferred system is a rapid thermal cycling device using capillary tubes and hot air temperature control. See, for example, United States Patent No. 5,455,175, herein incorporated by reference. Because of the low heat capacity of air and the thin walls and high surface area of capillary tubes, small volume samples could be cycled quickly. The total amplification time for 30 cycles is reduced to 15 minutes with excellent results.

The use of capillaries with forced air heating allows precise control of sample temperature at a speed not possible with other designs. For example, sample temperature verses time plots in capillaries show sharp spikes at denaturation and annealing temperatures, whereas several seconds are required for all of the sample to reach equilibrium in conical plastic tubes. Wittwer, CT, GB Reed and KM Ririe, Rapid cycle DNA amplification, in K Mullis, F Ferre, and R Gibbs (Eds.), The polymerase chain reaction, Springer-Verlag, Deerfield Beach, FL. pp. 174-181, 1994; Wittwer, CT, BC Marshall, GB Reed, and JL Cherry, Rapid cycle allele-specific amplification: studies with the cystic fibrosis delta F508 locus, Clin. Chem., 39:804-809, 1993. Rapid temperature cycling with minimal annealing and denaturation times improves quantitative PCR and increases the discrimination of allele specific amplification. Weis, JH, SS Tan, BK Martin, and CT Wittwer, Detection of rare mRNA species via quantitative RT-PCR, Trends in Genetics, 8:263-4, 1992; Tan ST and JH Weis, Development of a sensitive reverse transcriptase PCR assay, RT-RPCR, utilizing rapid cycle times, PCR Meth. and Appl. 2:137-143, 1992. Rapid cycling for cycle sequencing reduces sequencing artifacts and minimizes "shadow banding" in dinucleotide repeat amplifications. Swerdlow H, K Dew-Jager and RF Gesteland, Rapid cycle sequencing in

an air thermal cycler, BioTechniques 15:512-519, 1993; Odelberg SJ and R White, A method for accurate amplification of polymorphic CA-repeat sequences, PCR Meth. Appl. 3:7-12, 1993. For long PCR, yield is improved when the sample is exposed as little as possible to high denaturation temperatures. Gustafson CE, RA Alm and TJ Trust, Effect of heat denaturation of target DNA on the PCR amplification. Gene 23:241-244, 1993. The RapidCycler®, developed by Idaho Technology, is an example of a rapid thermal cycling device. The LightCycler® (Roche Diagnostics, Indianapolis, IN) is a rapid temperature cycler with a fluorimeter, wherein light emitting diodes are used for excitation and photodiodes are used for detection.

10                   The present invention is directed to methods for automating detection nucleic acids with real time PCR. While these algorithms may be applied to any amplification system, it is preferred to integrate these algorithms into the LightCycler® platform. These analysis routines are triggered by the completion of rapid thermal cycling for “hands off” amplification, analysis, and final results presentation in a total of  
15 less than 15 min. The analysis routines take from <1 second for detection and quantification to <10 seconds for genotyping. LabView (National Instruments, Austin, TX), a graphical programming language, is preferred for LightCycler® instrument control. The LightCycler® is a PC-based instrument.

                  Perhaps the most basic analysis of real time PCR data is a judgement of  
20 whether a targeted nucleic acid is present. If the nucleic acid is present, further quantification and genotyping may take place. In many cases, a yes/no judgement is all that is needed. For example, one may want to determine whether E. coli 0157:H7 is in a sample of hamburger, whether anthrax is on a swab from a soldier; or whether hepatitis C is in a unit of blood. Real time PCR can improve yes/no detection over end point PCR  
25 assays because fluorescence is acquired at each cycle.

                  Inspection of cycle verses fluorescence data from positive and negative real time PCR runs (see Figs. 1h and 1i) suggests that discrimination is simple. The positive samples increase with cycle number while the negative samples remain at baseline. A trained observer expects positive samples to follow an S-shape curve,  
30 beginning with a baseline, followed by an exponential segment, and finishing with a plateau. The expected curve is similar to the logistic model for population growth, where the rate of growth is proportional to both the population size  $y$  and to the difference  $L-y$ ,

where  $L$  is the maximum population that can be supported. For small  $y$ , growth is exponential, but as  $y$  nears  $L$  the growth rate approaches zero. An example of logistic growth is shown in Fig. 2.

Although intuitively simple, accurately discriminating between positive and negative samples is not easy in practice. The simplest approach is to set a horizontal fluorescence threshold as a discriminator between positive and negative samples. This works best with a stable baseline (between and within samples) and a known fluorescence intensity that correlates with "positive." Although this method will work on obvious samples (e.g. Figs. 1h and 1i), a more robust algorithm is desired that will work under a wider variety of conditions. For example, the baseline may drift and the fluorescence intensity may vary greatly between different samples and probe techniques. Thus, the present invention is directed to a method that will: (1) automatically identify the baseline, (2) use the baseline variance to establish a confidence region, and (3) call each sample positive or negative based on the relationship of the confidence region to the fluorescence data.

Figs. 3a-f display various types of amplification curves, all of which have been observed in LightCycler® runs. Figs. 3a and b show curves from samples that are negative with no template present. The fluorescence scales in Figs. 3a and b are magnified (compared to Figs. 3c-f) to demonstrate the baseline drift and to provide algorithms capable of being independent of the fluorescence intensity. There is always some baseline drift during cycling. This drift usually is greatest at the beginning of cycling but later levels off, and may be either downward (Fig. 3a) or upward (Fig. 3b). This baseline drift of negative reactions must be distinguished from positive reactions of either low copy numbers (Fig. 3c) or high copy numbers (Fig. 3d) of starting template. The method needs to work with various probe designs, including exonuclease (Fig. 3e) and hybridization (Fig. 3f) probes.

Automatic identification of the background is surprisingly difficult. In prior art methods, the baseline is determined as a function of measured fluorescence at a fixed range of cycles near the beginning of amplification. However, selection of a fixed range of cycles is not adequate because both downward drift (Fig. 3a) and high copy (Fig. 3d) amplifications may be incorrectly called.

### ***Confidence Band Analysis***

In the present invention, the background is identified by analyzing the fluorescent measurements over a wide range of amplification cycles. Preferably, the background is identified by selecting the sliding window (Fig. 4) with the shallowest slope. That is, calculate the slope at each cycle by linear regression of the local neighborhood (for example, a 7 point sliding window). The window with the slope of lowest absolute value (least difference from zero) defines the background region. Once the background region has been identified, the variation of these background points about their regression line (the square root of the mean square error) is multiplied by a constant to determine a confidence band. This confidence band will have a slope near zero and is extrapolated across all cycles. If the fluorescence of the last cycle is within the confidence band it is negative, if it is outside the band it is positive. Fig. 5 demonstrates both cases.

This algorithm should work well in most cases. However, with the high copy fluorescence curve type (Fig. 3d), the shallowest slope might be found at early cycles (resulting in a correct positive call) or at late cycles (resulting in an incorrect negative call). This exception may be handled by analyzing the curve shape. In a well-behaved amplification, the expected amplification curve shape is ordered by cycle number as follows:

1. Minimum fluorescence
2. Maximum second derivative ( $F''$ )
3. Maximum first derivative ( $F'$ )
4. Minimum second derivative ( $F''$ )
5. Maximum fluorescence

This gives the characteristic S-curve shape expected during PCR (Fig. 6A). The maximum slope (first derivative) is obtained from the sliding window analysis already performed for background identification. Preferably, the second derivatives are calculated by a 3-point sliding window linear regression of the first derivatives. If the curve shape is well behaved (that is, if looking at a graph of Fig. 6, and reading from lowest to highest cycle number, the features occur in the order listed above), then the

background is only selected from sliding windows centered at cycle numbers less than the second derivative maximum. This solves the potential analysis problem with Fig. 3d. In other preferred embodiments, cycle numbers less than the first derivative maximum or cycle numbers less than the second derivative minimum may be used. It will be further understood that any cycle number between the second derivative maximum and the second derivative minimum is a suitable cutoff cycle for use with this technique and is within the scope of this invention.

Another method is to compare the cycle with the greatest fluorescence (which is not necessarily the last cycle) to the confidence band. This is especially suited for hybridization probes that may decrease in fluorescence with extensive cycling, such as seen in Fig. 3f. The cycle with the greatest fluorescence only should be used when the curve shape is well behaved, in order to prevent false positive calls with downward drifts, such as shown in Fig. 3a.

The variables to optimize for automatic detection are: 1) the window size for the first derivative estimate, 2) the window size for the second derivative estimate, and 3) the confidence band factor. A reasonable value for the first derivative window size is 7, although 3, 5, 9, and 11 are also quite useful. For the second derivative the preferred window size is 3, but 5, and 7 have also proven to be useful values. A preferred confidence band factor is 20. As the first derivative window size increases the variance estimate is more accurate, but the edge cycles (beginning and ending) are lost.

This algorithm is best understood by referring to the fluorescence verses cycle test result plot shown in Figs. 7-11. The input data consist of one fluorescence value for each cycle of amplification, shown as the closed white circles. Let this equal array  $Y_i$ , where  $i$  is the cycle number and  $N$  is the total number of cycles. The detection criteria are:

$A$  = the number of fluorescence values used to determine the first derivatives. It is convenient to use odd numbers, so that the first derivatives correspond to integer cycle numbers. As discussed above, reasonable values include 3, 5, 7, 9, and 11. Preferably, 7 is used as the first derivative window size.

B = the number of first derivative values used to determine the second derivatives. Again, it is convenient to use odd numbers, so that the second derivative values also correspond to integer cycle numbers. Reasonable values include 3, 5, and 7, with 3 being the preferred value.

5

C = the confidence band factor. This factor determines the confidence band by multiplying it by a variance measure, preferably the square root of the mean square error.

10

The first step is to calculate the first and second derivatives. Although there are many ways to accomplish this, a preferred method is to determine the first derivatives as the slope of a linear regression line through A points, and assigning the value to the central cycle number. Some cycles on either edge cannot be assigned first derivatives, but first derivatives can be provided for cycles  $(A + 1)/2$  through  $N - (A - 1)/2$ . Similarly, the second derivatives are calculated as the slope of the first derivative points and assigned to cycles  $(A + 1)/2 + (B - 1)/2$  through  $[N - (A - 1)/2] - (B - 1)/2$ . Calculation of the first and second derivatives provide arrays  $Y'_i$  and  $Y''_i$ , with some edge values missing. In Fig. 7, the first and second derivatives are displayed as open black circles and closed black circles, respectively.

20

The next step is to determine whether the fluorescence curve has a well-behaved shape. As discussed above, the well-behaved shape occurs when the cycles with minimum fluorescence, maximum second derivative, maximum first derivative, minimum second derivative, and maximum fluorescence occur in that order, from low to high cycle number.

25

The baseline is then determined. If the fluorescence curve does not have the expected shape, the cycle whose first derivative is closest to zero is used. If the fluorescence curve has a well-behaved shape, the cycle whose first derivative is closest to zero chosen from among all cycles prior to the cycle with the maximum second derivative (again, any cycle between the maximum second derivative and the minimum second derivative may also be used as the cutoff cycle number). The baseline is drawn through the fluorescence value of the chosen cycle with a slope of its first derivative. In

30

Fig. 7, the A points contributing to the first derivative calculation for the baseline are displayed as large black dots connected by a line.

The next step is to determine the test point cycle, that is, the cycle used to compare against the baseline for determining a positive or negative result. If the curve is not well-behaved, the test point is the last cycle. If the fluorescence curve is well-behaved, the test point is the cycle with fluorescence farthest from the baseline. The test point fluorescence of a negative sample can be predicted as the intersection of the baseline with the test point cycle.

Next, a confidence interval can be determined about the predicted negative test point. Preferably, this is done by finding the square root of the mean square error about the baseline of A points used to determine the baseline. This is multiplied by C. The product is added to the predicted negative test point to get the upper fluorescence limit of the confidence interval and is subtracted from the predicted negative test point to get the lower limit of the confidence band. These limits are shown on Fig. 7 as two solid horizontal lines.

The final step is to declare the sample positive or negative. If the test point fluorescence is outside of the confidence interval, the sample is positive. If it is within the interval, the sample is negative. Figs. 7 and 8 are samples which are positive, while Figs. 9-11 are negative samples.

### ***Melting Temperature Analysis***

In another embodiment, the "positive" calls generated by the above method are further confirmed by automatic feedback of the melting temperature ( $T_m$ ) value of the amplified product. This additional confirmation is possible as long as the hybridized and non-hybridized states of the probe can be distinguished by changes in fluorescence signal, as with dsDNA dyes and hybridization probes. The  $T_m$  of an amplified product can be determined as follows: at a predetermined and/or dynamically chosen amplification cycle, fluorescence is monitored between extension and denaturation (or annealing and denaturation, in the case of a two-step amplification process). In an illustrated embodiment, the fluorescence is monitored at each  $0.1^\circ\text{C}$  temperature increment. However, the monitoring may occur at smaller or larger increments. This monitoring will provide a melting profile of the amplified product.

-15-

Alternatively, a  $T_m$  can be obtained by adding a separate melting process at the end of the amplification cycle, during which fluorescence is continuously monitored and a melting profile is obtained. The minimum (or maximum, depending on whether the probe design produces a melting peak/valley), of the derivative of this melting profile will determine the  $T_m$ . The  $T_m$  value will then be compared with the known  $T_m$  of the target analyte, and if the two values are in concordance, a verified positive call is made. If they are discordant, then a “positive” call is not verified. This technique may be used, for example, to identify situations where a locus other than the target locus was amplified or where primer dimers were produced.

10

Although the invention has been described in detail with reference to preferred embodiments, variations and modifications exist within the scope and spirit of the invention as described and defined in the following claims.